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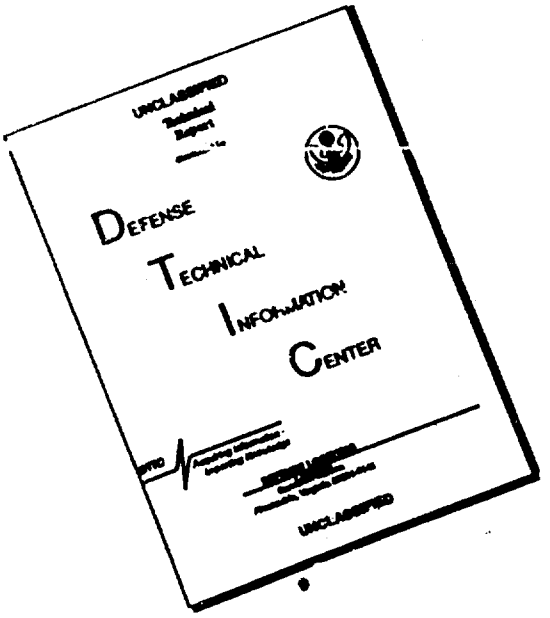

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Utilization of Indirect Hemagglutination Reaction for Determination of Botulinal Toxins

Report II

Modification Method of Indirect Hemagglutination Reaction and Its Comparative Evaluation with Some Tests Employed for Detection of Botulinal Toxins

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In report I*) we discussed some questions in connection with the influence of certain physical and chemical factors on the sensitivity, specificity and rapidity of the indirect hemagglutination reaction. On the basis of conducted investigations we prepared a modification of the test in question pertinently to determination of botulinal toxins. Thus, in the current report we discuss a variant of the indirect hemagglutination reaction, as developed by us, also the evaluation data of the method which was compared with the biological test on mice, as well as compared with the hemagglutination reaction in modification of RYTSAI (correctly: RYCAJ) and with the MINERVIN method. Since, in using this method to detect botulinal

*) - For report I see Journal of Microbiology, Epidemiology and Immunology, 1960, No.3.

toxins type A and B, we obtained analogous results, thus we quote in this report only investigation data on the toxin type B.

We used in the experiments liquid botulinal toxin type B, which in 1 ml contained 50 Dlm (correctly: MLD) for white mice weighing 10 to 12 gm, and antibotulinal sera type A (15,625 BU/ml), also type B (8,064.5 BU/ml). In addition, we considered essential for our variant method the following constituents: sheep erythrocytes, tannic acid solution and normal serum of rabbits.

Sheep erythrocytes were obtained in a usual way; they were washed five times with a physiological solution of table salt (0.85%, pH 7.2) prior to carrying out the experiments, until the fluid above the sedimentation became completely colorless. A 3% suspension of the precipitate was prepared with the same solution.

A solution of tannic acid in a concentration of 0.1% was prepared with the physiological solution of table salt (pH 7.0) immediately before the experimentations.

We inactivated fresh rabbits' serum (without any traces of hemolysis) in water bath at 56°C in 30 minutes, then adsorbed it at room temperature (19 to 25°C) with sheep erythrocytes and diluted it with a physiological solution of table salt (pH 7.0) in proportion of 1:100.

On the day of experiments, the toxin was adsorbed with sheep erythrocytes at room temperature in 10 minutes, then the mixture was centrifuged and the obtained centrifugate was pumped off; the latter was used subsequently for a preparation of a series of dilutions for which a normal rabbit serum was used as diluting agent.

The sheep erythrocytes were processed with tannic acid. Thus, 1 volume of a 3% suspension of sheep erythrocytes was added to 1 volume of 0.1 °/oo solution of tannic acid, then the mixture was kept in water bath at 37°C for 10 minutes and, subsequently, it was centrifuged. The residue was washed twice with a physiological solution of table salt (pH 7.2) and then it was resuspended in 1 volume of the physiological solution of table salt (pH 7.0). We effected the sensitization of sheep erythrocytes in this way: to 1 ml of tannized erythrocytes we added 16,129 BU (2 ml) of antibotulinal serum type B and 8 ml of physiological solution of table salt (pH 6.4); next, we agitated the test tubes and placed them for 15 minutes under thermostatic control at 37°C. After we centrifuged the mixture, the residue (erythrocytes) was washed twice with a normal rabbit serum and then it was resuspended in 1 ml of the same serum.

The planning of the test was reduced to this course: to each test tube with 0.5 ml of suitable dilution of botulinal toxin we added 0.1 ml of erythrocytes sensitized with antibotulinal serum; we agitated this and placed it under thermostatic control at 37°C until the completion time of reaction in control test tubes, which, we considered, usually required 1½ to 2 hours time.

Every investigation was accompanied by the following controls:

1) 0.5 ml of the least diluted botulinal toxin + 0.1 ml of nonsensitized sheep erythrocytes;

2) 0.5 ml of normal rabbit serum + 0.1 ml of nonsensitized sheep erythrocytes;

3) 0.5 ml of normal rabbit serum + 0.1 ml of sensitized sheep erythrocytes.

For research on white mice we prepared suitable dilutions of botulinal toxin in a physiological solution of table salt; the toxin was administered in 1 ml doses to animals by intraperitoneal injections. Since we carried out our experiments with toxins well known, we omitted the neutralization test.

For determination of the phagocytic index according to the method of MINERVIN, the botulinal toxin type B was diluted with a physiological solution of table salt (0.85%, pH 7.0) and it was poured per 1 volume in each into three series of test tubes. Then, to each test tube of the first series we added one volume of the physiological solution (pH 7.0); to the second series - antibotulinal toxin type A, and to the third series - type B serum. We used for control purposes the least diluted botulinal toxin heated in boiling water bath for 20 minutes. The inactivated toxin was also poured into each of the three series of test tubes, to which we added physiological solution and corresponding antibotulinal sera. The test tubes were agitated and placed under thermostatic control at 37°C; then, after 40 minutes, we removed from each 1 volume of contents and added 1 volume of citrated blood of rabbits (2 volumes of blood + 1 volume of 1.6% solution of sodium citrate), we agitated this and left for 20 minutes at 37°C. Subsequently, we put into each test tube 1 volume of suspension of Staphylococcus aureus (strain 209) that contained 2 million microbes in 1 ml and we placed the test tubes again under thermostatic control for 20

Table 1

Sensitivity Evaluation Results of the Biological Test and of the Modification of Indirect Hemagglutination Reaction in Determination of Botuline Toxin

Dilution of botuline toxin	Results of tests on white mice				Results of indirect hemagglutination reaction
	1st day	2nd day	3rd day	4th day	
1:10	OOOO	ØØØØ			
1:50	ØOOO	ØOO	OO	ØØ	
1:100	OOOO	OOOO	OOOO	ØOOO	++++
1:200	Alive	for	10 days		++++
1:400	"	"	" "		++++
1:800	"	"	" "		++

Symbols: O mouse alive; Ø dead mouse.

Table 2

Evaluation of Sensitivity of Indirect Hemagglutination Reaction in Modification of RYTSAI Processed by Us

Serum / Toxin's dilution	Modification of RYTSAI			Author's modification		
	Type A	Type B	Physiological solution	Type A	Type B	Physiological solution
1:100	—	++++	—	-	++++	-
1:200	—	++++	—	-	++++	-
1:400	—	++	—	-	++++	-
1:800	—	—	—	-	+++	-
Toxin heated at 100° for 20 minutes	-	-	-	-	-	-
Normal rabbit serum	-	-	-	-	-	-

minutes. Next, we obtained from each test tube two smear preparations, which were later fixed with NIKIFOROV'S mixture and we stained them with azure-eosin dye. We computed in each preparation the number of microbic cells in 25 neutrophiles. In order to determine the phagocytic index, we divided by 50 the general quantity of microbes phagocyted with 50 leukocytes.

We submit in Table 1 the comparative results of indirect hemagglutination reaction and of biological test.

In carrying out the biological test of botulinal toxin, we diluted the latter with the physiological solution (pH 7.0) to 1:10, 1:50, 1:100 and 1:200 for indirect hemagglutination reaction, using a normal rabbit serum with a ratio of 1:10, 1:50, 1:100, 1:200, 1:400 and 1:800. The results obtained indicate that the hemagglutination reaction is considerably more (approximately 8 times) sensitive than the biological test on mice; also, the test time in determination of minimal amounts of botulinal toxin with the aid of indirect hemagglutination reaction is approximately 24 times shorter than the test time in determination on white mice.

Our conditions of conducting a test of a comparative evaluation of the indirect hemagglutination reaction were exactly the same as those in the modification of RYTSAI. Also, the initial components used were the same; the only difference was in the method itself. In numerous determinations of the indirect hemagglutination reaction in both modifications we noticed that the titer in modification of RYTSAI varied within a range of 2 to 3 dilutions, whereas in our modification the hemagglutination titer seldom changed, even in a

single dilution. We frequently noticed a change in the indicia of agglutination in a test tube that contained a minimum concentration of toxin determinable.

In Table 2, which reflects one of our experiments, we submit data showing that the hemagglutination reaction in our modification is more sensitive than the reaction of RYTSAI.

In a comparative evaluation of the sensitivity of indirect hemagglutination reaction (in our modification) with the modified method of MINERVIN, we also used the same specimens of the toxin and of antibotulinal sera. The investigation reflected in Table 3 shows that the indirect hemagglutination reaction exceeded in sensitivity the modified method of MINERVIN. The exact results from determination of the phagocytic index, which were obtained with dilution of toxin at 1:100, and the indirect hemagglutination reaction, permitted to detect and to differentiate exactly the A and B types of the toxin in a dilution at 1:400.

Speaking about sensitivity of the method, we have in mind a determination of minimal concentrations of the toxin in a few milliliters of fluid. If we take into account that with modification of the MINERVIN method we used 0.025 ml of each dilution of the toxin and 0.5 ml in indirect hemagglutination reaction, it becomes obvious that the first method is considerably more sensitive. Of course, a detection of botulinal toxin in such small quantities as 0.025 ml is of no specific importance in a practical application, but it is important to be able to detect a minimal concentration of botulinal toxin that doesn't change with relation to the volume of the examined

Table 3

Results from Testing the Sensitivity of the Modified Method of MINERVIN and that of the Indirect Hemagglutination Reaction in Determination of Botuline Toxin Type B

Serum / Toxin's dilution	MINERVIN'S method			Indirect hemagglutination reaction		
	Type A	Type B	Physio-logical solution	Type A	Type B	Physiological solution
1:100	1.2	2.66	0.9	-	++++	-
1:200	1.57	2	1.3	-	++++	-
1:400	2.37	2.74	2.24	-	++++	-
1:800	2.6	2.58	2.14	-	++	-
1:1,000	2.24	2.21	2.66	-	—	-
1:10,000	2.71	2.98	3.2	-	—	-
1:100,000	2.69	2.4	2.45	-	—	-
1:100 heated for 20 minutes at 100°C	2.54	2.96	2.2	-	-	-
Normal rabbit serum	-	-	-	-	-	-

Table 4

Comparative Evaluation of the Sensitivity Methods in Determination of Botuline Toxin

Method	Sensitivity (in Dlm)	Average error (± m)	Average quadratic divergence	Reliability of distinctions *) of two indices

fluid used in analysis. The investigations proved that an increased quantity of the investigated material did not increase the sensitivity of the modified MINERVIN'S method as used, while the sensitivity of the indirect hemagglutination reaction did increase with the increased volume of the investigated material.

The statistical analysis of obtained results proved the following. In the course of titration of the toxin in 10 experiments on animals (120 white mice), we determined that one minimal fatal dose of the toxin corresponded to the average dilution of 1:90. Hence, we regarded a higher dilution of the toxin as a definite part of Dlm. For example, a dilution of the toxin at 1:1,600 corresponded to 0.05 Dlm and a dilution at 1:800 corresponded to 0.11 Dlm, etc. Thus, the reliability of differences obtained in comparable experiments with respect to the sensitivity of our method versus other methods (biological test on white mice, RYTSAI'S method and modified test of MINERVIN) we determined by analyzing an average error according to the formula: $\frac{M-M_1}{\sqrt{m^2 + m_1^2}}$; where M and M_1 are comparable arithmetical means; where m and m_1 are their average errors respectively. We considered as important differences only those that exceeded the minimal error in diversity (i.e. they were greater and occurred not less than 3 times).

Judging from the t values submitted in Table 4, the differences in sensitivity of our method, as compared with the biological test on white mice, with the method of RYTSAI and with the modified method of MINERVIN, can be evaluated as substantial and reliable.

Table 5

Determination of Botuline Toxin in Solutions with Diverse Concentrations of Table Salt in Rendition of the Modified Method of MINERVIN and that of the Indirect Hemagglutination Reaction

Concentration of table salt (in %)	Serum	Indirect Hemagglutination Reaction with Type B Toxin					Modified MINERVIN'S method with type B toxin	
		Diluted				Inactivated	Diluted 1:100	Inactivated
		1:100	1:200	1:400	1:800			
0.85	Type A	-	-	-	-	-	2	
	Type B	++++	++++	++++	++++	-	3.66	
	0.85% solution of table salt	-	-	-	-	-	1.32	3.71
2	Type A	-	-	-	-	-	0.94	
	Type B	++++	++++	++++	++++	-	0.62	
	2% solution of table salt	-	-	-	-	-	0.63	0.42
4	Type A	-	-	-	-	-	0.23	
	Type B	++++	++++	++	++	-	0.18	
	4% solution of table salt	-	-	-	-	-	0.4	0.2
5	Type A	-	-	-	-	-	0.14	
	Type B	++++	++++	++	++	-	0.13	
	5% solution of table salt	-	-	-	-	-	0.04	0.13
8	Type A	-	-	-	-	-	0.2	
	Type B	++++	+++	++	++	-	0.13	
	8% solution of table salt	-	-	-	-	-	0.2	0.1
10	Type A	-	-	-	-	-	0	
	Type B	++++	++	-	-	-	0	
	10% solution of table salt	-	-	-	-	-	0	0
15	Type A	-	-	-	-	-	0	
	Type B	+++	+++	-	-	-	0	
	15% solution of table salt	-	-	-	-	-	0	0

Symbols: 0 absence of phagocytosis; - negative reaction; ++, +++, ++++, various degrees of positive reactions.

Of greater practical importance is a study of the possibility of determination of botulinal toxin in various concentrations of table salt. Such study would be in connection with a possibility of poisoning with botulinal toxin after eating salted fish. The concentration of table salt in extracts made of strongly salted fish may reach to 8 or 10%.

Hence, we diluted botulinal toxin with a solution (pH 7.0) containing diverse contents of table salt from 0.8 to 15%. We used the same percentage contents of table salt for indirect hemagglutination reaction while diluting the toxin with normal rabbit serum.

Data presented in Table 5 indicate that determination of botulinal toxin with the modified method of MINERVIN is difficult, when the concentration of table salt approximates 2%. The indirect hemagglutination reaction enabled us to detect and to differentiate a type of botulinal toxin, when a concentration of table salt equaled 15%. As the concentration of table salt increased from 2 to 15%, the hemagglutination titer decreased gradually from 1:800 to 1:200. At the same time, we observed a gradual retardation in precipitation of erythrocytes.

Conclusions

1. The indirect hemagglutination reaction in our modification, used to determine botulinal toxin, exceeded in sensitivity the modification of RYTSAI, also the biological test on white mice and the modified method of MINERVIN.

2. The modified method of MINERVIN enabled us to determine

botulinal toxin in investigated fluid with the concentration of table salt not exceeding 2%; the indirect hemagglutination reaction in our modification permitted us to detect and to differentiate a type of botulinal toxin in a fluid with a concentration of table salt up to 15%.

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Summary (copied)

The author presents a modified technique of indirect hemagglutination reaction, as applied in the determination of botulinum toxin; he compares the sensitivity of this reaction with the biological test on white mice, indirect hemagglutination reaction (modified by RYTSAI) and the modified method of MINERVIN,

The data obtained demonstrate that the suggested modification of the indirect hemagglutination reaction permits to determine 1/8 MLD of botulinum toxin for white mice within 3 hours.

By the sensitivity and stability of its results the test surpasses the indirect hemagglutination according to RYTSAI and the modified MINERVIN'S method.